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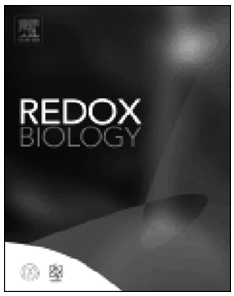

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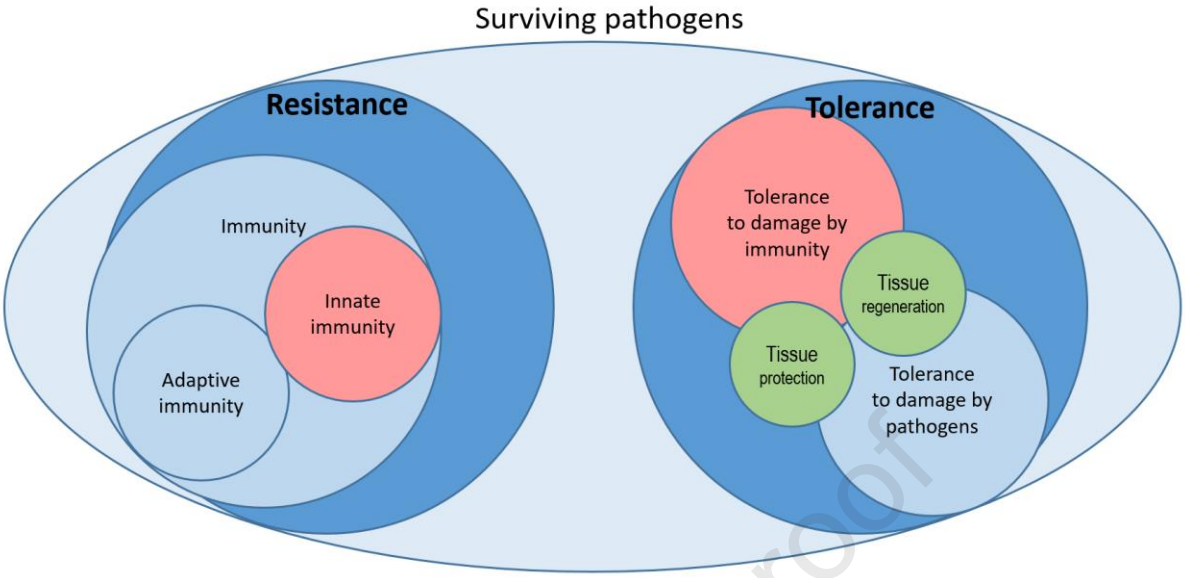
Abstract

It is thought that excessive production of reactive oxygen species (ROS) can be a causal component in many diseases, some of which have an inflammatory component. This led to an oversimplification whereby ROS are seen as inflammatory and antioxidants anti-inflammatory. This paper aims at reviewing some of the literature on thiols in host defense. The review will first summarize the mechanisms by which we survive infections by pathogens. Then we will consider how the redox field evolved from the concept of oxidative stress to that of redox regulation and how it intersects the field of innate immunity. A third section will analyze how an oversimplified oxidative stress theory of disease led to a hypothesis on the role of ROS and glutathione (GSH) in immunity, respectively as pro- and anti-inflammatory mediators. Finally, we will discuss some recent research and how to think out of the box of that oversimplification and link the role of thiols in redox regulation to the mechanisms by which we survive an infection outlined in the first section.

Key words

Glutathione; innate immunity; Nrf2; NF-kB; HIF-1α; inflammation

Graphical abstract



Surviving pathogens: resistance versus tolerance

To describe this concept, we need to take a step back and consider the whole picture rather than focusing on one the aspects of response to pathogens (typically, immunity and inflammation). Without mentioning the mechanical barriers (e.g. skin) or the mucociliary escalator, we deal with an infection by two very different mechanisms: resistance and tolerance [1-3].

Resistance, or pathogen control, consists in killing the pathogen or inhibiting its growth. This is the field of immunity, of which innate immunity and the inflammatory response are one arm which is activated early before adaptive immunity develops a more sophisticated and specific arsenal of antibody-producing B-lymphocytes-derived plasma cells and cytotoxic T cells [4]. Resistance is also mediated by phagocytes producing bactericidal ROS and activating interferon (IFN) pathways that result in inhibition of viral proliferation.

Tolerance, or damage control, on the other hand, has not been systematically studied but is well known in plant physiology [5], and consists of various mechanisms that limit the damage that pathogens causes to the host, for instance by the neutralization of toxins released by the infection. Tolerance was described for respiratory infections; for instance, tissue protection mechanisms that can protect from infection without affecting the pathogen load have been described in mice infected with *Legionella pneumophila*, influenza or both [6, 7]. Another example is how the host copes with malaria, not only by controlling the number of parasites but also by inducing heme oxygenase (HMOX1) that prevents the toxic effect of hemolysis-derived hemoglobin [8].

It is important to note, however, that if resistance and tolerance are completely different responses, their effector cells may be, in some cases, the same, and innate immunity was suggested to contribute to tissue repair and therefore tolerance [9, 10]. A further overlap between the two concepts is that tolerance can include mechanisms that protect not only against the damage caused by the pathogen but also against host-mediated toxicity, such as excessive inflammation [3]. Tolerance can be mediated by stress responses and tissue repair mechanisms [11]. For instance, in an infection by Gram-negative bacteria, exposure to bacterial lipopolysaccharide (LPS, endotoxin) can induce a form of tachyphylaxis known as “endotoxin tolerance” [12, 13]. This process switches off the inflammatory response and therefore further inflammation-induced tissue damage [12, 13]. Figure 1 provides a scheme of the main mechanisms of resistance and tolerance.

Innate immunity and inflammation

Innate immunity is triggered by pattern recognition receptors, such as the Toll-like receptors (TLRs) upon binding to pathogen-associated molecular patterns. In the case of Gram-negative bacteria, LPS binds TLR4 activating a signaling cascade that leads to the activation of NFkB and transcription of inflammatory genes [14, 15]. The products of these genes, such as inflammatory cytokines or inducible cyclooxygenase generating arachidonic acid metabolites, then augment vascular permeability and, through the action of chemokines, recruit inflammatory cells, like neutrophils and macrophages, to the site of infection. Neutrophils in particular, but also macrophages, produce reactive oxygen species (ROS) as part of their bactericidal armamentarium during the NADPH oxidase (NOX)-mediated "oxidative burst" [16], a mechanism of pathogen resistance also present in plants [17].

In the 1980s, it became clear that most of the innate immune mechanisms of resistance are mediated by proinflammatory mediators called cytokines, such as interleukins (IL)-1 IL -6 and tumor necrosis factor (TNF). One important aspect of innate immunity is that the same mechanisms that are essential in limiting infection can also induce tissue damage to the host through the inflammatory response. This is known as the "cytokine theory of disease" that led to the development of some of the most effective biologicals currently approved for use for chronic inflammatory diseases, such as anti-TNF and anti-IL-6 antibodies [18, 19].

Oxidative stress, antioxidants and immunity: the development of the concept of redox regulation

The popular view of the oxidative stress (OS) theory of disease is based on the idea that excessive production of reactive oxygen species (ROS) or insufficient antioxidant systems to cope with it are a causal component of many disease conditions [20]. The idea that free radicals can cause disease was first made explicit in a 1956 paper by Harman who put forward the "free radical theory of ageing" [21]. We have adopted the definition of "OS theory of disease" elsewhere [20], by analogy with other theories of disease that imply specific causal agents, like germs in the germ theory of disease [22] and inflammatory cytokines in the cytokine theory of disease [19]. However, by no means this implies that these theories are equal in terms of strength. In fact, while the germ theory of disease gained strength by the development of antibiotics and the cytokine theory by the approval of anti-cytokine antibodies (particularly anti-TNF and anti-IL-6), small antioxidants, acting to scavenge ROS, have not been approved yet for any indication. While, obviously, a theory can be perfectly valid even if it doesn't lead to any drug discovery, if should be noted that most studies hypothesizing a pathogenic role for OS, including the 1956 study by Harman [21], have more or less explicitly predicted that antioxidant treatments would be effective.

One of the possible reasons why the administration of antioxidants acting as ROS scavengers has not been successful is that ROS are not just toxic byproducts in the metabolism but also participate in the regulation of various signaling pathways, which defines as redox regulation [23]. Interestingly, this concept was first described when studying the role of ROS in infection and immunity. In 1991, a pioneering work by the laboratory of Patrick Bauerle showed that hydrogen peroxide activates the transcription factor NFkB, an effect antagonized by the antioxidant N-acetylcysteine (NAC) [24]. In the same year, the group of Anthony Fauci reported that NAC and GSH inhibit the transcriptional activity of the HIV promoter when this is activated with the inflammatory cytokine TNF [25]. Those two studies indicated that ROS and small-molecular weight thiols can affect the activation of the transcription factors regulating the expression of inflammatory genes as well as the action of inflammatory cytokines.

On one hand, this connection between inflammatory cytokines and GSH prompted many studies on the protective effect of thiol antioxidants in various models of cytokine-mediated disease [26-28]. These studies also showed an inhibitory effect of thiol antioxidants on TNF production *in vivo* [29]. As a result, many researchers in the field worked on the assumption that ROS are pro-inflammatory mediators and GSH (as well as other thiols) is an anti-inflammatory molecule. The idea was that inflammation is associated with increased ROS production that, in turn, would augment NFkB activation and cytokine production, thus exacerbating inflammation. However, despite the fact that most preclinical studies showed a protective effect of NAC, the level of evidence required for drug approval has never been reached in adequately powered randomized clinical trials. Clinical trials with NAC in acute respiratory distress syndrome (ARDS) never showed a level of evidence for efficacy sufficient to recommend it as a treatment (reviewed in [30]).

In his discussion on mechanistic reasoning and clinical trials, Howick described how lack of efficacy could be explained by the fact that the effects of a compound on patients are a combination of its action on the desired pharmacological target and other, often less well defined, mechanisms [31]. The lack of success of thiols and other ROS scavengers might be due the fact that ROS are not only pathogenic mediators but also essential in the regulation of signaling molecules. Likewise, GSH may not be only a ROS scavenger but also a regulator of signaling molecules that can modulate the function of several redox-regulated proteins via disulfide formation, including glutathionylation [23, 32]. Interestingly, many pathways and molecules associated with innate immunity are targets of glutathionylation [33]. These protein targets are at different levels of the inflammatory cascade, and include proteins interacting with Toll-like receptors, such as MyD88-adapter-like (MAL) [34], the p50 subunit of NF-kB [35] and the signal transducer and activator of transcription 3 (STAT3) [36]. Downstream of the inflammatory pathway, glutathionylation also occur at a specific cysteine of

interleukin 1 beta (IL-1β), affecting its biological activity [37]. Of note, susceptibility of free protein cysteines to oxidation, including glutathionylation, is an important factor that determines whether a specific protein is susceptible to regulation by GSH/GSSG or not. It should also be noted that, in redox regulation, GSH acts in concert with hydrogen peroxide that can directly catalyse protein glutathionylation or do so by changing the GSH/GSSG ratio, with a possible catalytic role of redox enzymes [38].

Over twenty years ago, Joe McCord acknowledged that ROS are not "as we once thought, just a toxic but unavoidable byproduct of oxygen metabolism" (this sentence was referring to superoxide radicals) [39], and others noted that ROS-producing NADPH oxidases (NOX) evolved soon after the appearance of oxygen in the atmosphere [40]. The concept of redox regulation assigns a central role to hydrogen peroxide as a signaling molecule [23, 32, 41].

If ROS are essential signalling molecules in innate immunity, then the question arises as to the role of GSH. Is GSH acting only as a ROS scavenger, detoxifying hydrogen peroxide through the action of GSH peroxidase, or also regulating signaling pathways where redox-sensitive proteins are implicated?

Glutathione in inflammatory cells: a signaling molecule or a scavenger?

Most of the studies on the anti-inflammatory role of GSH have been obtained using NAC as a precursor, or a mimic, of GSH. Consistently, most studies reported that NAC, *in vivo* or *in vitro*, inhibited the LPS-induced production of inflammatory cytokines, particularly TNF (see, for instance, [29, 42-47], although one study using the macrophage cell line J774 observed an inhibition of TNF production by NAC only at 1.5% oxygen, where ROS production is higher than under atmospheric oxygen concentrations (21%) [42].

The reports on the effects of NAC in models of TNF-mediated inflammation are not consistent in their conclusions. While some studies showed that administration of NAC protects from LPS-induced lethality and pulmonary damage [27, 28, 48], others reported that the effect is dose-dependent, and that high NAC doses can increase LPS mortality [49]. There is, however, a consensus for an inhibitory effect on the production of inflammatory cytokines *in vitro* and *in vivo*, although there are reports, for instance, of NAC increasing LPS-induced IL-1b production *in vitro* [50], with the possibility that the effect of NAC may be concentration-dependent and observed only at the highest doses [46, 47].

Induction of inflammation by LPS does not have a consistent effect of GSH levels in all experimental models. While some studies reported a decrease [51-53] and others an increase [46, 54], most studies reported a lack of effect [55-63].

It is important to note, however, that NAC cannot be considered a specific tool to study the role of endogenous GSH. While it is a precursor of GSH synthesis, it is also a ROS scavenger itself and a potent reducing agent [64], and the mechanism of this is debated [65].

Other tools used to investigate the role of GSH are buthionine-SR-sulfoximine (BSO), that inhibits GSH synthesis from cysteine [66], or diethyl maleate (DEM), an electrophile that reacts with GSH by direct conjugation thus depleting it [67, 68]. However, DEM is less specific [68, 69]. Diethyl maleate can also be contaminated with dimethylfumarate [69], which is an Nrf2 inducer [70]. Thus, BSO should be considered a reasonably specific tool to investigate the role of endogenous GSH. Table 1 summarizes the results of several studies investigating the effect of BSO on LPS-induced inflammatory cytokines. It can be seen that there is no consensus, so that the results from each study cannot be extrapolated outside the specific experimental conditions of cell culture, LPS concentrations, length of treatments and others. Thus, one cannot generalize the assumption that endogenous GSH is a negative regulator of the inflammatory response, and this may explain the lack of translational success, in terms of drugs approved so far, of the use of GSH and its precursors as therapeutic agents in inflammatory disease.

Could NFkB be important in resistance and Nrf2 in tolerance, and what are the other players?

The activation of NFkB by LPS is acknowledged as a key pathway in innate immunity, the early-onset mechanism of resistance through pathogen control [71, 72]. In fact, NFkB target genes (such as cytokines, cyclooxygenase, nitric oxide synthase) induce the classical inflammatory response in terms of recruitment of neutrophils and macrophages, activation of their killing mechanisms and antigen presentation that also contribute to the activation of adaptive immunity.

On the other hand, activation of Nrf2 is often associated with an anti-inflammatory response [73], part of which mediated by carbon monoxide produced by HMOX1 [74]. The anti-inflammatory action of carbon monoxide has been observed in lipopolysaccharide-induced models of septic shock [75] as well as in tissue-specific models of inflammatory disease [76, 77].

Activation of Nrf2 also results in the inhibition of the production of inflammatory cytokines [78, 79]. The main role of Nrf2, however, is adapting the organism to survive against oxidants and other electrophiles [80, 81] including various xenobiotics [82]. The activation of Nrf2 by LPS has been

reported in different models [61, 83, 84], and several papers demonstrated the key role of Nrf2-induced HMOX1 not only in the tolerance to malaria mentioned above but also in protecting from LPS lethality or polymicrobial sepsis [84-86]. When considering HMOX1 as a Nrf2 target gene in the context of inflammation, however, it is important to note that HMOX1 is not solely controlled by Nrf2 [74], and its induction by LPS or other Toll-like receptor agonists has a different pattern compared to other Nrf2 target genes [87, 88]. Finally, Nrf2 is negatively regulated by Glycogen Synthase Kinase-3β (GSK3beta) [80, 89, 90] and this may contribute to the diminished LPS-induced cytokine production observed with GSK3beta inhibitors [91].

In addition to NFkB and Nrf2, hypoxia-inducible factor 1αa (HIF-1α) should be considered. This transcription factor is important in adapting to low oxygen concentrations leading to physiological responses that promote oxygen delivery through the induction of erythropoietin (EPO) and vascular endothelial growth factor (VEGF), as well as cellular responses leading to a switch to glycolysis [92, 93]. In particular, EPO has been shown to act as a tissue-protective cytokine that limits damage by inflammatory mediators and promotes tissue repair [94, 95]. In the context of this review, EPO administration protects from lethality and organ damage in animal models of sepsis [96, 97] and improves survival of mice with cerebral malaria without affecting parasitemia [98].

Therefore, the HIF-1α pathway could also play a role in tolerance, by protecting the host from the damage induced by the pathogen and/or by the immune response to it. Although the main role of HIF-1α is oxygen sensing, rather than ROS sensing as in the case of Nrf2, several reports indicate that it is redox regulated, and for this reason we discuss it here. In particular, ROS can stabilize HIF-1α by inactivating the prolyl hydroxylase responsible for its destabilization [99] as well as by increasing its mRNA levels via NFkB [100]. ROS can also stimulate the transcription of several HIF-1α target genes [101]. Conversely, antioxidant molecules have the opposite effect, with NAC and Vitamin C reducing HIF-1α levels [102] and ebselen and pyrrolidine dithiocarbamate inhibiting ROS-mediated activation of the transcription of EPO [101].

Whether HIF-1α has a pro- or anti-inflammatory role is debated. However, while in the context of cancer HIF-1α has been considered pro-inflammatory [103], in the context of innate immunity many data suggest the opposite. In fact, administration of chemical HIF-1α stabilizers inhibits NFkB activation and inflammatory cytokine production [104-106] [107], and protects from TNF-induced tissue damage [108, 109] as well as *Clostridium difficile*-induced intestinal injury [110].

Finally, although not discussed in the present review, it should be mentioned that the three transcription factors I discussed are not independent from each other. It is important to highlight that Nrf2 and NFkB are not separate and independent. Studies have shown that Nrf2 can inhibit

NFkB activation [86, 111]. Signaling via HIF-1α is promoted by Nrf2, as shown by experiments where Nrf2 or its inhibitor were knocked out [112-114].

Stabilization of HIF-1α by the prolyl hydroxylase inhibitor dimethyloxalylglycine inhibits the production of inflammatory cytokines in models of inflammation and endotoxic/septic shock [107, 109, 115] although it should be noted that in specific microenvironments, such as in cancer, a pro-inflammatory role of HIF-1α has been described [116, 117].

Conclusions

From the literature reviewed here, one could come to the general conclusion that NFkB has a key role in resistance while Nrf2 and HIF-1a are more important in tolerance and that, in general, most antioxidants have an inhibitory effect, and ROS a stimulating one, on these transcription factors. In this context, GSH does not inhibit the expression of all the genes associated with innate immunity but rather facilitates the induction via TLR4 of a subset of genes associated with the antiviral response while those for most inflammatory cytokines are independent of GSH, at least in the mouse macrophage experimental model discussed in this review.

We might therefore conclude that, while a schematic view of NFkB mediating resistance and Nrf2/HIF-1α mediating tolerance may hold, GSH-mediated redox regulation is not a master switch in the system but rather a signaling mechanism that fine tunes those systems by adding an additional control to regulated subset of genes [61].

Figure 2 is an attempt to describe the different roles of transcription factors and of the effectors of resistance and tolerance, as well as indicating where redox regulation can act, either through ROS or GSH/GSSG, or both. It should be noted that the role of ROS as signaling molecules vs. toxic mediators depends on their intracellular concentrations [23].

The fact that GSH regulates the response to pathogens by a more complex mechanism that just acting as a ROS scavenger can explain why, in some instances, thiol antioxidants were not effective in clinical trials despite a very convincing mechanistic theory. It may not be surprising that administration of these reducing agents could in fact interfere with key signaling pathways, including those outlined in this review.

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Table 1. Effect of BSO in models of LPS-induced inflammatory cytokines

Model	Result	Ref	Year
vivo mice	TNF ↑	[29]	1992
vitro U373 astrocytoma	IL-8 ↑	[118]	1997
vivo mice	TNF ≈	[119]	1999
vitro alveolar macrophages	TNF, IL-8 ↑	[120]	1999
vivo mice	TNF ↓	[121]	1999
vitro THP1 monocytic cells	IL-12 ↓	[122]	2001
vitro epitelial cells	TNF ↑	[123]*	2002
vitro epitelial cells	TNF ↑	[124]*	2002
vitro epitelial cells	TNF, IL-1b, IL-6 ↑	[125]*	2002
vitro epitelial cells	TNF, IL-1b, IL-6 ↑	[126]*	2002
vitro epitelial cells	TNF, IL-1b, IL-6 ↑	[127]*	2002
vitro U937 monocytic cells	TNF ↑	[128]	2005
vitro THP1 monocytic cells	TNF ≈	[129]	2008
vitro epithelial cells	TNF ↑	[130]	2008
ex vivo mouse peritoneal macrophages	IL-6 ↓	[131]	2009
vitro epithelial cells	TNF ↑	[132]*	2011
vitro dendritic cells	IL-12, IL-27 ↓	[133]	2011
vitro RAW264 macrophages	IL-1b ↓	[134]	2017
vitro RAW264 macrophages	TNF ≈, IL-1b ↓	[61]	2017
vivo rat	IL-1b, IL-6, fever ↓	[135]	2017
vitro RAW264 macrophages	TNF ≈	[136]	2018
vitro RAW264 macrophages	IL-1b, IL-6 ≈	[62]	2018
vitro RAW264 macrophages	TNF ↓	[137]	2021

↑, increase; ↓ decrease; ≈ no effect. *These different publications report the same experiment and should not be considered replications of published data.

Figures

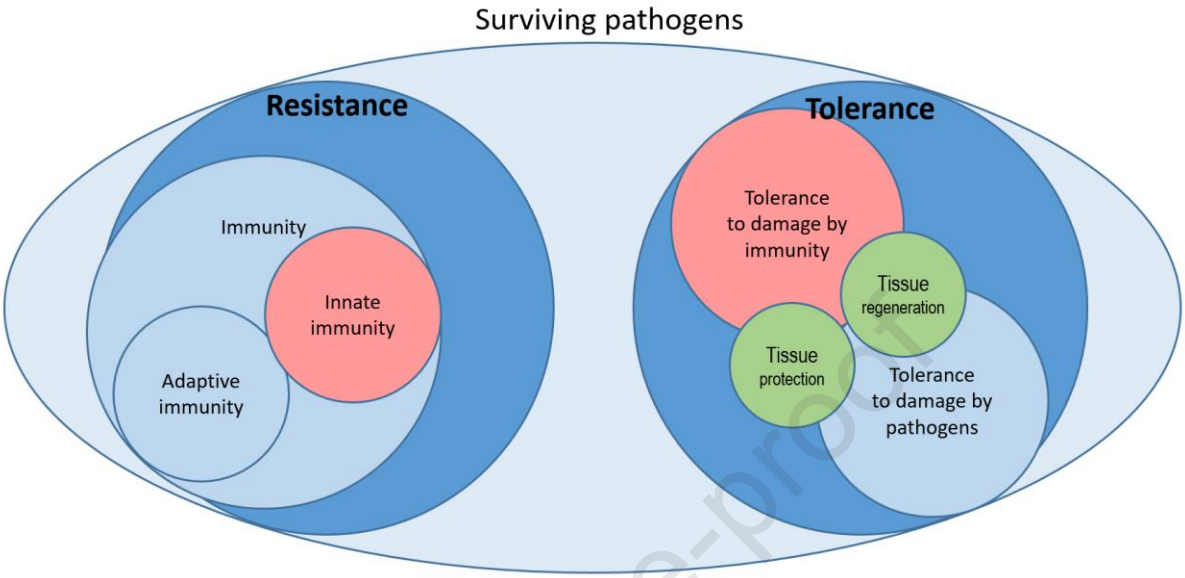

Figure 1. Resistance and tolerance as the two mechanisms to survive infections

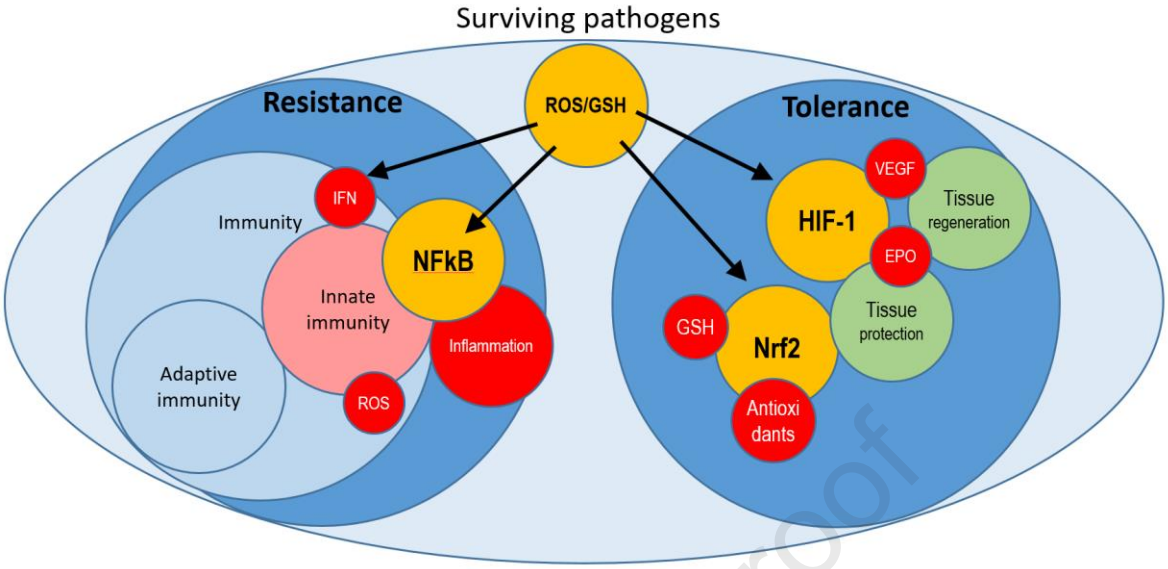

Figure 2. Regulators (yellow) and effectors (red) of resistance and tolerance. Left: innate immunity controls pathogens by ROS production by phagocytes (mainly antibacterial) and upregulation of the IFN pathway (mainly antiviral) as well as activation of the inflammatory response through activation of NFkB. Right: tolerance includes tissue protection by Nrf2-mediated upregulation of antioxidant systems and HIF-1-mediated induction of tissue-protective cytokines (e.g. EPO) as well as promotion of tissue repair through induction of growth factors (e.g. VEGF). Please note the dual role of ROS and GSH both as effectors and regulators.

Figure legends

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Redox regulation of immunity and the role of small molecular weight thiols

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Professor Pietro Ghezzi
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